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Upregulation of CRISP-3 and kallikrein in stallion seminal plasma is associated with poor tolerance of cooled storage

Short title: Stallion seminal plasma proteins

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Contents

For unknown reasons, stallion fertility and sperm longevity during cooled storage of semen vary markedly between individuals. Spermatozoa from individual stallions react differently to the presence, or the removal, of seminal plasma (SP). The aim was to evaluate differences in protein content in stallion seminal plasma with either a positive or a negative effect on sperm chromatin integrity during storage. Stallion semen samples from different ejaculate fractions were stored at 5˚C for 24h. Sperm survival was assessed after storage using a sperm chromatin structure assay. Protein expression in SP with either positive or negative effects on sperm survival during storage was studied using two-dimensional differential gel electrophoresis and liquid chromatography - mass spectrometry. Lower sperm chromatin integrity was associated with upregulation of the proteins kallikrein, CRISP-3, and HSP-1, while higher chromatin integrity was associated with upregulation of TIMP-2. In the sperm-rich fractions, kallikrein and CRISP-3 differed significantly between SP samples with differing effects on sperm chromatin integrity. In the sperm-poor fractions, TIMP-2 and HSP-1 differed significantly between the two SP groups. Differences in the seminal plasma proteome are associated with sperm longevity during cooled storage.

Keywords:

seminal plasma protein, spermatozoa, sperm chromatin integrity, sperm storage, stallion

Introduction

Stallion fertility and sperm longevity during cooled storage of semen vary markedly between individuals, but the reasons for this are largely unknown. The composition of

stallion seminal plasma (SP) varies between stallions and different fractions of the ejaculate (Kareskoski *et al*. 2010a, Kareskoski *et al.* 2010b). The ejaculate fractions differ in sperm concentration and semen volume, with the first few jets, the sperm-rich fractions, containing the main portion of spermatozoa. The last sperm-poor fractions consist of secretions from the seminal vesicles with low sperm concentration (Tischner *et al*. 1974, Kosiniak 1975, Varner *et al*. 1987, Magistrini *et al.* 1995).

Reducing the proportion of SP to less than 10-20% for cooled storage of semen is beneficial for sperm survival during storage (Jasko *et al*. 1991, Pruitt *et al*. 1993, Braun *et al*. 1994, Todd *et al*. 2001, Alghamdi *et al*. 2002, Love *et al*. 2005), but the effects of SP on sperm survival can vary between the fractions of the ejaculate. Most studies have reported higher sperm motility and quality in sperm-rich fractions as compared to the sperm-poor fractions (Varner *et al.* 1987, Sieme *et al*. 2004, Kareskoski *et al.* 2011).

Variation between stallions in the effects of SP on spermatozoa is evident. It has been reported that spermatozoa from individual stallions react differently to the presence, or the removal, of SP (Padilla & Foote 1991, Jasko *et al*. 1992, Akcay *et al.* 2006). Exchange studies, where spermatozoa from one stallion were stored in another stallion's SP, have also been carried out. Seminal plasma from some stallions seem to have a less negative, or even a beneficial, effect on sperm survival during storage (Aurich *et al.* 1996). It has been recommended that stallions producing semen with poor tolerance to cooling and storage should be evaluated for the effects of their SP on semen quality, and the semen processing technique modified accordingly (Brinsko *et al.* 2000, Love *et al.* 2005).

Stallion SP contains several different types of proteins. The main SP proteins are proteins carrying fibronectin type II (Fn-2) modules, cysteine-rich secretory proteins (CRISPs), and spermadhesins. The suggested functions of SP proteins include involvement in several essential steps preceding fertilization, such as regulating capacitation, establishment of the oviductal sperm reservoir, modulation of the uterine immune response, sperm transport in the female genital tract, and gamete interaction and fusion (reviewed by Töpfer-Petersen *et al.* 2005).

The aim of this study was to evaluate protein expression in stallion SP with either positive or negative effects on sperm survival during storage. Two-dimensional differential gel electrophoresis and liquid chromatography - mass spectrometry was used for proteomic analysis and protein identification.

Materials and methods

Animals

Semen was collected during one breeding season from 43 stallions (ages 4 to 23 years) residing at nine stud farms in Finland. Twenty stallions were Standardbred trotters (ages 4 - 23 years), 17 Finnhorses (ages 8 - 23 years), four warmblood riding horses (ages 9 - 19 years) and two were ponies (ages 8 and 9 years). The breeding history and current use of the stallions was variable, with the stallions serving from 2 to 150 (median 14) mares during the study season. Most of the stallions (33 stallions) were used for collection of both fresh and transported semen, and four stallions were also used for natural breeding. The eight stallions that were selected for proteomic

analyses were aged 10 to 23 years, and were used for commercial breeding regularly during the year of the study. Ethical approval was not required for this study according to the Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013).

Semen collection

The ejaculatory jets were collected as three to four fractions using one of the three types of artificial vaginas (AV): an open-ended artificial vagina equipped with a collection bag attached to a funnel (Krakow AV; Tischner et al. 1974) a Missouri AV with a collection bag (Kareskoski *et al.* 2011) or a computer-controlled fractioning phantom with an integrated AV (Equidame phantom, Haico Oy, Loimaa, Finland; Lindeberg et al. 1999), depending on stallion preference and stud farm. To provide comparable samples, fractions with the highest and lowest sperm concentrations within each ejaculate were included in the statistical analyses. The mean concentrations for the fractions were 300.0 x 10^6 and 54.0 x 10^6 sperm/ml, respectively, with no significant differences in sperm concentration in fractions collected with the different methods. Of the stallions selected for proteomic analyses, all were collected using the open-ended AV, except for stallion E, which was collected with the modified closed AV.

Semen processing and storage

After semen collection, any gel was removed if present, and the volume of each fraction measured. The sperm concentration in each fraction was determined using a Bürker counting chamber. For the sperm chromatin structure assay (SCSA), a sample of 2 - 10 x 10^6 spermatozoa from each fraction of raw semen was pipetted into

cryovials and TNE-buffer (9.48 g Tris-HCl, 52.6 NaCl, 2.23 g disodium-EDTA, aqua ster. ad 600 mL, pH 7.4) was added (ad 1.5 mL in each vial). The SCSA samples were placed in liquid nitrogen vapor (3cm above the liquid surface) for 10min, and then plunged into liquid nitrogen.

Each fraction was divided into two parts: one-half was centrifuged for the preparation of SP (SP samples), and the other half was processed for cooled storage (semen samples). A portion (10%) of the volume of each fraction was combined to form a sample representing the whole ejaculate (WE). For cooled storage, semen samples were extended in a semen:extender ratio of 1:1 using skim milk extender (Kenney *et al.* 1975). Each fraction of the semen samples was divided into two centrifuge tubes and centrifuged at 500 x g (10 min). After removal of the major part (about 5% was left) of the supernatant (i.e. extender and SP), the sperm pellet was extended in either: a) skim milk extender only (these samples stored without SP were named SP0 samples), or b) a combination of supernatant and skim milk extender (these samples stored with SP were named SP1). The final sperm concentration was 50 x 10^6 sperm/ml. The final SP to extender ratio in the SP1 samples was 1:2. The semen samples were stored in 1-mL vials at 5°C for 24h. After the 24-h cooled storage, 5 x 10^6 spermatozoa from each semen sample were pipetted into cryovials for SCSA, and TNE-buffer was added (ad 1.5 mL in each vial). The samples were placed in liquid nitrogen vapor (3cm above the liquid surface) for 10min and plunged into liquid nitrogen.

The SP samples were centrifuged at 4000 x g (15 min), and the supernatant was filtered using 0.45-µm filters (Millex-HV, Millipore, Billerica, MA, USA). Protease

inhibitor (Trasylol, 10000 Kallikrein Inactivator Units (KIU)/mL, Bayer Schering Pharma, Berlin, Germany) was added to each sample (500 KIU/mL of SP) to decrease proteolytic activity on enzymes and other proteins. The SP samples were stored frozen in 1-mL aliquots in -75°C until analyzed.

Sperm chromatin structure assay

Sperm chromatin stability of sperm from both the raw and stored semen samples was measured as the susceptibility of sperm DNA to denaturation using the sperm chromatin structure assay (SCSA) as described by Evenson *et al.* (1999). Acridine orange was added to each thawed sample after exposure to an acid detergent solution that induces partial DNA denaturation *in situ*. Flow cytometry was then used to measure either red (abnormal sperm with denatured DNA) or green (normal sperm) fluorescence). Sperm DNA fragmentation index (DFI) was assessed by calculating the amount of red fluorescence divided by the total (red plus green) fluorescence, indicating the amount of denatured sperm DNA over the total DNA in each sperm cell.

Selection of samples for proteomic analyses according to sperm survival during cooled storage with or without seminal plasma

The samples of eight stallions were chosen for the proteomic analyses based on the effect of SP on sperm chromatin integrity during storage, represented here by the difference in DFI (diff-DFI) between semen samples stored with SP and the samples stored without SP (diff-DFI = SP1 – SP0). The samples with the lowest and the highest diff-DFI (-5.9 to +0.8% and +10.6 to +13.5%, respectively) of all the stallions were selected, and the samples were grouped into two groups according to the diff-

DFI. The effect of SP on sperm chromatin integrity was negative in the samples with high diff-DFI (group HIGH DIFF), and the effect of SP was positive or neutral in the samples with low diff-DFI (group LOW DIFF). The diff-DFI values varied significantly between the groups according to Student's t-test (p=0.008) (Table 1).

Sample preparation for proteomic analysis and CyDye labelling

Seminal plasma samples were prepared to proteomic analysis by precipitation with 2D Clean Up-kit and solubilized in 50 µl of labeling buffer (7 M Urea, 2 M Thiourea, 4 % CHAPS, 30 mM Tris). The concentration of proteins in the samples was measured by using 2D Quant Kit according to manufacturer's instructions. The samples were then labeled with Cy2, Cy3 and Cy5 dyes (CyDye DIGE Fluor minimal dyes) according to Ettan two-dimensional difference gel electrophoresis (DIGE) protocol. Briefly, 50 µg of protein from each sample were labeled with 400 pmol of the Cy3 and Cy5 dyes. Equal amount aliquots of each sample were combined to establish internal standard labeled with Cy2 dye. The labeling reaction was incubated 30 minutes on ice in the dark and halted by adding of 1 mM lysine to the reaction following 10 minutes incubation as earlier. All materials and equipment by GE Healthcare, Piscataway, NJ, USA.

Two-dimensional difference gel electrophoresis

The labeled proteins were analyzed by two-dimensional difference gel electrophoresis (2D-DIGE) as described earlier (Ünlü *et al.* 1997). An immobilized pH gradient (IPG) strip (24 cm, pH 3-10, nonlinear, GE Healthcare, Piscataway, NJ, USA) was used for isoelectric focusing. IPG strips were loaded with 150 µg of protein in total by using cup-loading method. Isoelectric focusing was performed using IPGPhor (GE

Healthcare, Piscataway, NJ, USA) at 20°C as follows: 3h at 150 V, 3h at 300 V, then linear ramping to 10 000 V and 10 000 V for 50 000 Vh with maximum current per strip being 75 µA. After focusing the isoelectric strips were prepared for the second dimension gels by incubation in I (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS), 0.2% bromophenol blue, with added 10mg/ml dithiothreitol (DTT)) equilibrium buffer solution for 15 minutes. This was followed by equilibration in a II (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.2% bromophenol blue, supplemented with 25mg/ml iodoacetamide) buffer solution for another 15 minutes. The prepared IPG strips were then placed on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and sealed with overlay agarose (Bio-Rad, Hercules, CA, USA). Electrophoresis was initiated at 50 V for 30 minutes, which was followed by 400 V for 3 hours. The gels were scanned between low-fluorescence glass plates using an FLA-5100 laser scanner (Fujifilm, Tokyo, Japan) at wavelengths 473 (for Cy2), 532 (for Cy3) and 635 nm (Cy5) using voltages of 420, 410 and 400 V. After scanning the gels were stained by PlusOne silver staining kit (GE Healthcare, Piscataway, NJ, USA) without aldehyde.

Image analysis and data processing

The gel images were analyzed and statistically assessed using DeCyder 7.0 software (GE Healthcare, Piscataway, NJ, USA). First the gels were automatically analyzed using the batch processor function to normalize the Cy2, Cy3 and Cy5 images from each gel. Spot volumes were calculated and compared to Cy2 volumes (internal standard) to correct the inter-gel variations. In the biological variation module, the Cy2 images of all gels in groups were matched and the spot volumes compared. Approximately 1000 separate spots were detected on each gel. Protein spots

demonstrating a minimum of 1.5-fold difference in average spot volume ratios between groups using Student's *t* test (*p*-value less than 0.05) were chosen for identification.

Protein identification

Protein spots of interest were manually excised from the gel and digested in-gel using trypsin (Trypsin Gold, Promega, Madison, WI, USA) as described earlier (Shevchenko *et al.* 1996; Jensen *et al*. 1998). The peptides were extracted, vacuum dried (Vacufuge Plus vacuum concentrator, Eppendorf, Hamburg, Germany), centrifuged (Eppendorf microcentrifuge, Eppendorf, Hamburg, Germany) and re-dissolved in 20 µl 5% formic acid. Digested peptides were de-salted using C18 Zip-Tips (Millipore, Burlington, MA, USA) according to the manufacturer's instructions. The resulting peptides were identified by fragment ion analysis with LC-MS/MS using Ultimate 3000 nano-LC system (Dionex, Sunnyvale, CA, USA) and QSTAR Elite hybrid quadruple TOF mass spectrometer (Applied Bioystems/MDS Sciex, Foster City, CA, USA) with nano-ESI ionization. The identification of proteins was performed using the local Mascot version 2.2 (Matrix Science, London, UK) against the in-house database with the following criteria: one missed cleavage allowed, carbamidomethyl modification of cysteine as a fixed modification, and oxidation of methionine as a variable modification.

Results

Approximately 1000 different proteins were expressed in the 2D-DIGE analysis. Only four proteins were significantly ($p<0.05$) differentially expressed in SP of the different storage groups (SP0 and SP1). The identified proteins were tissue inhibitor

of metalloproteinase-2 (TIMP-2), horse seminal protein-1 (HSP-1), kallikrein, and cysteine-rich secretory protein-3 (CRISP-3).

In the sperm-rich fractions, kallikrein and CRISP-3 were upregulated in SP samples with high diff-DFI (i.e. a low rate of sperm survival during storage with SP). In the sperm-poor fractions, TIMP-2 was upregulated in SP samples with low diff-DFI (i.e. a high rate of sperm survival during storage), and HSP-1 was upregulated in samples with high diff-DFI (Table 2).

Discussion

Poor sperm survival during cooled storage in the presence of SP was associated with upregulation of the SP proteins kallikrein, CRISP-3, and HSP-1, while better sperm survival was associated with upregulation of TIMP-2. Sperm survival was evaluated using a chromatin structure assay, which has been shown to be correlated with sperm survival and fertility in stallions (Love *et al.* 2005, Johannisson *et al.*2018). The effects of SP on sperm motility can differ from those on sperm chromatin integrity, and SP can damage chromatin markedly while motility is maintained (Morrell *et al.* 2012). In this study, the stallions were categorized according to diff-DFI alone, because of the correlation between fertility and sperm chromatin integrity (Love 2005, Morrell *et al*. 2008).

Increased levels of CRISP-3 were associated with high diff-DFI, which reflects poor sperm survival during storage when SP is present. This is not necessarily directly linked to decreased fertility, which is a multifactorial trait. Most studies on CRISP-3 in stallion semen associate upregulation of the protein with increased fertility,

whereas in our study upregulation was associated with decreased sperm survival during storage. Based on genomic studies, CRISP-3 is associated with stallion fertility and semen quality traits (Hamann *et al.* 2007, Novak *et al.* 2010, and Gottschalk *et al.* 2016). In raw semen, high levels of CRISP-3 in SP result in higher sperm motility and improved semen quality index (Restrepo *et al.* 2019), but in our study, higher levels of CRISP-3 decreased sperm longevity during storage.

Proteins that are correlated to fertility in raw or frozen semen may not be associated with sperm longevity during cooled storage. During storage, proteolytic changes in SP may alter the protein composition, as SP contains several different proteases and protease inhibitors (Laflamme & Wolfner 2013). At present, the magnitude and significance of these proteolytic alterations are not known. The relationship between SP proteins and sperm survival during cooled storage has not previously been studied, except for our earlier study on SP proteins and cooled semen storage for 24h where we found no significant correlation between the level of HSP-1 and sperm motility parameters (Kareskoski *et al*. 2011). The protein HSP-1 has been shown to protect other proteins against thermal and chemical stress conditions (Sankhala *et al.* 2012), and to prevent lipid peroxidation under oxidative stress (Kumar & Swamy 2016). In our study, the levels of HSP-1 itself were higher in samples with poor sperm survival.

The mechanisms behind the association of these proteins with semen quality, freezability and fertility are largely unknown. The CRISP-3 protein is an androgen-regulated protein expressed only in postpubertal horses (Fedorka *et al.* 2017). Associations between this protein and semen quality traits are not necessarily direct and causal, but rather indirect correlations of androgen-dependent traits. A possible

295 role of CRISP-3 in the regulation of oxidative pathways in semen has been proposed
296 (Restrepo *et al.* 2019), based on the high content of cysteine residues in CRISP-3
297 (Udby *et al*. 2005) and the contribution of these residues to oxidative reactions
298 (Requejo *et al.* 2010). In human semen, seminal vesicular fluid makes SP a zinc-
299 binding medium after ejaculation. This can deplete spermatozoa of zinc and affect
300 sperm chromatin decondensation (Björndahl & Kvist 2010), and CRISP proteins
301 could also enhance the appearance of DNA lesions in sperm (Rodrigues *et al*. 2016).
302 This could explain the association between CRISP-3 and DNA injuries shown in our
303 study.
304
305 Kallikrein was associated with high diff-DFI and DNA injuries during cooled storage.
306 It is a protease similar to prostate-specific antigen (PSA) produced by the prostate
307 gland in humans. Kallikrein and HSP-1 have earlier been shown to be negatively
308 correlated to freezability (Jobim *et al.* 2011) and pregnancy rate, especially at low
309 sperm concentrations and low dilution rates, when the amount of SP is high in relation
310 to the number of spermatozoa. It was suggested that these proteins bind to
311 spermatozoa and reduce their ability to fertilize the ovum (Novak *et al*. 2010). In our
312 study, the negative effects of kallikrein on sperm chromatin integrity were found in
313 the sperm-rich fraction of the ejaculate.
314
315 Both matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are found in SP
316 (Baumgart *et al*. 2002). In our study, less sperm DNA damage during storage was
317 associated with higher levels of TIMP-2. Similarly, high levels of TIMP-2 are
318 associated with low levels of sperm DNA fragmentation in men (Belardin *et al.* 2019)
319 and high fertility in bulls (McCauley *et al.* 2001, Viana *et al*. 2018).

In the sperm-rich fractions, kallikrein and CRISP-3 differed significantly between HIGH DIFF and LOW DIFF stallions. In the sperm-poor fractions, TIMP-2 and HSP-1 differed significantly between these groups. The composition of these fractions differs to some extent, but in our earlier study, there were no significant differences in the amount of HSP-1 or CRISP-3 between fractions (Kareskoski *et al.* 2010b). However, both sperm chromatin integrity (Björndahl & Kvist 2010) and the function of some SP proteins, such as kallikrein and CRISP-3 (Carvalho *et al.* 2002, Schambony *et al*. 1998), are Zn-dependent, and hence the levels of Zn may affect the observed fraction differences. The levels of Zn in stallion ejaculates are correlated to sperm concentration (Pesch *et al*. 2006), which suggests that the seminal vesicles, contributing mostly to the sperm-poor fractions of the ejaculate (Magistrini *et al.* 1995), are not the main source of Zn in stallion SP. In primates, Zn is mostly derived from the prostate gland, and is therefore found in the sperm-rich fractions (Srivastava *et al.* 1984). The Zn-regulated proteins kallikrein and CRISP-3 were significant for sperm chromatin integrity in only the sperm-rich fraction in our study.

Conclusions

Poor sperm survival during cooled storage when SP was present was associated with upregulation of the SP proteins kallikrein, CRISP-3, and HSP-1, while better sperm survival was associated with upregulation of TIMP-2.

Conflict of interest statement

None to declare.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the corresponding author. The data are not publicly available due to privacy restrictions, e.g. their containing information that could compromise the privacy of research participants.

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601 Table 1. DNA fragmentation index (DFI) in semen samples stored at 5˚C for 24h with
602 or without seminal plasma (SP). Difference in DFI = (DFI when stored with SP) –
603 (DFI when stored without SP).

Stallion	**DFI (%) before storage**	**DFI (%) after storage**		
		Stored without SP	Stored with SP	Difference in DFI
A	10.3	11.8	5.8	-5.9
B	22.7	29.7	28.5	-1.2
C	11.1	13.5	13.5	-0.01
D	17.6	25.0	25.8	0,8
E	17.3	21.6	32.2	10.6
F	22.4	30.6	41.6	11.0
G	11.1	7.6	19.5	11.9
H	35.8	33.1	46.5	13.5

604

605

606 Table 2. Seminal plasma proteins with significant (p=0.05) upregulation in samples with a low rate of sperm survival during cooled storage with
607 seminal plasma (evaluated as changes in sperm chromatin integrity), compared with samples with a high rate of sperm survival during cooled
608 storage. (CRISP-3 = cysteine-rich secretory protein-3; TIMP-2 = tissue inhibitor of metalloproteinase-2; HSP-1 = horse seminal protein-1; pI =
609 isoelectric point).

Gel ID	Identified protein	Accession number	pI	Molecular weight (kDa)	Sequence coverage (%)	MASCOT score	Upregulation in
Seminal plasma in sperm-rich fractions							
6	Kallikrein	18250639	5.4	21.4	13.1	71	Low sperm survival
5	CRISP-3	3023562	7.4	27.0	21.2	111	Low sperm survival
Seminal plasma in sperm-poor fractions							
2	TIMP-2	135853	7.4	24.0	7.3	60	High sperm survival
5	HSP-1	1168021	8.2	14.0	42.9	112	Low sperm survival

610